MAJOR ARTICLE

Serologic Correlates of Protection against Enterotoxigenic *Escherichia coli* Diarrhea

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Background. We conducted a nested case-control study in 397 rural Egyptian children <36 months of age to assess the correlation between serum levels of antibodies against toxin and colonization factors (CFs) and the risk of homologous enterotoxigenic *Escherichia coli* (ETEC) diarrhea.

Methods. Active case detection was performed via semiweekly home visits, and blood was obtained at 3-month intervals. After each serosurvey, case subjects were selected from children experiencing a CF antigen (CFA)/I–, CFA/II-, CFA/IV-, or heat-labile enterotoxin (LT)–ETEC diarrheal episode during the subsequent 3 months. Up to 5 control subjects per case subject were selected from children who did not experience an ETEC diarrheal episode during the corresponding interval. Serum titers of immunoglobulin G antibodies against CFA/I, coli surface antigen (CS) 3, CS6, and LT were measured by enzyme-linked immunosorbant assay.

Results. The distribution of serum titers of LT, CS3, and CS6 antibodies did not differ between the case and control subjects. For children <18 months of age, serum titers of CFA/I antibody were inversely related to the risk of CFA/I-ETEC diarrhea; reciprocal serum titers of CFA/I antibody ⩾76 were associated with a 77% reduction in the odds of CFA/I-ETEC diarrhea.

Conclusion. Induction of reciprocal serum titers of antibodies against CFA/I within or above the 76–186 range should be further evaluated as a predictor for assessment of the ability of candidate vaccines to protect against CFA/I-ETEC diarrhea.

Enterotoxigenic *Escherichia coli* (ETEC) strains are well recognized as the principal cause of bacterial diarrhea during early childhood in resource-limited countries and among travelers visiting such settings [1–8]. Animal models and studies in humans have indicated that antibodies against surface-exposed ETEC colonization factors (CFs) and, less conclusively, antibodies against heat-labile enterotoxin (LT) play a role in protection against symptomatic disease [9–16]. Whether controlled ETEC challenge studies in adults mimic disease from natural exposure and result in immunity similar to that observed in settings where ETEC is endemic is largely unknown. Furthermore, it is not clear whether antibody levels in serum correlate well with levels of mucosal antibodies in the small bowel, where ETEC colonization occurs. If there is a correlation, it would be useful to know the approximate antibody level in serum that is associated with protection, because the methods required for measurement in serum are relatively easy, compared with those required for the direct measurement of levels of antibodies in intestinal se-

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cretions. If serologic correlates of protection against disease can be defined, such measures would facilitate the initial phases of evaluation of new ETEC vaccines by enabling the determination of the extent to which these candidate vaccines are capable of stimulating responses of a magnitude that predicts protection against disease. In the present study, we ascertain whether certain levels of CF antigen (CFA)/I, coli surface antigen (CS) 3, CS6, and LT antibodies correlate with protection against disease that results from infection with a homologous ETEC strain.

SUBJECTS, MATERIALS, AND METHODS

Enrollment and surveillance. The present study was conducted in 2 villages in the Nile Delta, as described elsewhere [7]. Briefly, in February 1995, after a census, 186 children <24 months of age were selected for enrollment. Thereafter, 211 newborns were enrolled, creating a total study cohort of 397 children. The children were followed until 36 months of age or February 1998. Written, informed consent was obtained from a parent or guardian of each child before enrollment. To detect ETEC diarrhea, 2 home visits were made each week. Whenever loose or liquid stool was reported, a rectal swab and a stool specimen were obtained from the child, for microbiological evaluation.

Beginning in July 1996, 5 serosurveys were conducted at 3-month intervals of all children ⩾6 months of age. Whenever possible, ~200–500 μL of blood was drawn into heparinized capillary tubes or capillary serum separators. When capillary tubes were used, whole blood was diluted 1:10 in PBS and centrifuged, to separate the serum. When serum separators were used, serum was obtained after centrifugation and stored without dilution. Serum specimens were stored at the US Navy Medical Research Unit No. 3 laboratory (Cairo, Egypt) at −20°C until testing. The human-use guidelines of the US Departments of Defense and Health and Human Services were strictly followed in the conduct of this study.

Laboratory methods. To identify ETEC, rectal swabs were plated on MacConkey's agar and grown overnight at 37°C. Up to 5 lactose-positive colonies per specimen were evaluated for both LT and heat-stable enterotoxin (ST) production, by LT GM1-ganglioside ELISA and ST inhibition GM1 ELISA [17, 18]. Colonies that were positive for either LT or ST were tested for the presence of a CF by use of a colony dot-blot assay that was based on a panel of 12 monoclonal antibodies that specifically detect CFA/I, CFA/II (CS1, CS2, and CS3), CFA/III (i.e., CS8), CFA/IV (CS4, CS5, and CS6), CS7, CS12, CS14, and CS17, as described elsewhere [19, 20].

Titers of serum IgG antibody against the B subunit of LT (LT_B) were measured by use of the GM1 ELISA method, and titers of serum IgG antibodies against CFs were measured by use of ELISA methods described elsewhere [21–23]. For CFA/II and CFA/IV, titers of IgG antibodies against purified CS3 and CS6, respectively, were evaluated, because these are the common antigens expressed for these CFs. Serum specimens were 3-fold serially diluted (initial dilution for LT_B, 1:500; initial dilution for CFA/I, CS3, and CS6, 1:20) in microtiter plates. Each serum specimen was assigned an antibody titer that was the geometric mean of measurements performed on the same specimen on different days. For undetectable titers, a value of one-half of the initial dilution was assigned as the final antibody titer.

Definitions. A diarrheal day was defined as the passage of either ⩾3 loose or liquid stools during any 24-h period (in addition, for breast-fed infants, the mother had to state that the stools were less formed or more frequent than usual) or ⩾1 loose or liquid stool with the presence of visible blood. A diarrheal episode was considered to be terminated when it was followed by ⩾3 consecutive nondiarrheal days. An ETEC diarrheal episode was defined as an episode in which ⩾1 lactose-fermenting colony from a fecal specimen obtained during the episode tested positive for either LT or ST. An ETEC diarrheal episode was classified as LT-ETEC if any colony tested positive for LT and all colonies tested negative for ST and was classified as ST-ETEC if any colony tested positive for ST and all colonies tested negative for LT. An ETEC diarrheal episode was classified as LTST-ETEC if the same colony tested positive for both LT and ST. Episodes for which the expression of CFA/I, CFA/II (CS1, CS2, and/or CS3), or CFA/IV (CS4, CS5, and/or CS6) was detected were classified as either CFA/I-, CFA/II-, or CFA/IV-ETEC diarrheal episodes, respectively.

Analytic methods. We employed a nested case-control design to assess whether levels of antibodies were inversely related to the risk of ETEC diarrhea. During early infancy, children have passively acquired serum maternal antibodies in addition to serum antibodies that result from natural ETEC infections. Because these maternal serum antibodies are not expected to be associated with the mucosal antibody levels in the small bowel that result from enteric exposure to ETEC, we restricted the analysis to children ⩾9 months of age, to minimize the detection of maternal antibodies. The choice of a conservative age cutoff of 9 months was based on the fact that maternal antibodies begin to wane at ~6 months of age and might still be present at low levels at this age. For the measurement of antibody titers, serum specimens were selected from the start of each 3-month interval.

Case subjects were children who experienced an ETEC diarrheal episode in which the infecting strain expressed either LT, CFA/I, CFA/II, or CFA/IV. Episodes in which *Shigella* species, *Campylobacter* species, rotavirus, or astrovirus were isolated in addition to ETEC were excluded, because it was not possible to attribute the symptoms of diarrhea to ETEC infection only. Anamnestic serum IgG antibody responses to ETEC infection have been shown to be extremely rapid in settings where ETEC is endemic [24]. To reduce the possibility that

Table 1. Clinical characteristics of case subjects who experienced an enterotoxigenic *Escherichia coli* (ETEC) diarrheal episode during which colonization factor antigen (CFA)/I, CFA/II, CFA/IV, or heat-labile enterotoxin (LT) was expressed.

Characteristic	CFA/I (n = 21)	CFA/II (n = 22)	CFA/IV (n = 23)	LT[a] (n = 51)
Toxin phenotype				
ST only	18 (86)	14 (64)	19 (83)	0 (0)
LTST	3 (14)	8 (36)	3 (13)	0 (0)
LT only	0 (0)	0 (0)	1 (4)	51 (100)
Age at onset of episode, mean ± SD, months	14.6 ± 4.5[b]	21.2 ± 7.4	22.6 ± 7.6	19.1 ± 8.1
Vomiting	2 (10)	1 (5)	1 (4)	4 (8)
Fever	11 (52)	11 (50)	11 (48)	19 (37)
Bloody stool	0 (0)	0 (0)	0 (0)	3 (6)
Maximum loose stools,[c] mean ± SD	8.8 ± 4.3[d]	6.3 ± 2.0	6.2 ± 2.3	6.3 ± 3.1
Episode duration, mean ± SD, days	2.7 ± 2.3	2.1 ± 2.1	1.2 ± 1.3	2.1 ± 2.6
ORS[e]	9 (43)	3 (14)	6 (26)	10 (20)
Took antibiotics	3 (14)	2 (9)	1 (4)	4 (8)

NOTE. Data are no. (%) of case subjects, unless otherwise noted. ST, heat-stable enterotoxin.

[a] Refers to LT-ETEC diarrheal episodes for which the ETEC isolates did not express a detectable CF.

[b] $P < .01$, for the comparison of CFA/I-ETEC diarrheal episodes to all others.

[c] Maximum no. of loose stools during any 24-h period during the episode.

[d] $P < .05$, for the comparison of CFA/I-ETEC diarrheal episodes to all others.

[e] Oral rehydration solution provided.

preclinical ETEC infections, by causing a primary or anamnestic serum immune response before the onset of diarrheal symptoms, would confound our results, case subjects with an onset of ETEC diarrhea within 3 days of serum collection were excluded. Whenever there were multiple episodes of homologous ETEC diarrhea between 2 serosurveys, the first episode was selected as the case.

Control subjects were selected from children who did not experience an ETEC diarrheal episode between 2 serosurveys. Control subjects might have experienced diarrheal episodes in which pathogens other than ETEC were isolated. In this setting, because the risk of infection with ETEC varies significantly depending on the season, it was necessary to ensure that exposure to ETEC was the same for both the case and control subjects [7]. To control for this seasonal variation, the control subjects were matched within the 3-month intervals. In addition to seasonal variation, the risk of infection with ETEC decreases with age, and the ideal study design would use a matching strategy based on both age and calendar time [7, 8, 25]. However, because matching on both variables would have limited the number of potential matched control subjects, the case and control subjects were matched on calendar time only. Within each 3-month interval, the control subjects were randomly selected on the basis of frequency, with up to 5 control subjects matched for each case subject.

Failure to analyze the results of studies designed as matched case-control studies by use of matched analytic methods can introduce bias into the interpretation of the results [26, 27]. In the present study, because the control subjects were matched to the case subjects for each serosurvey on the basis of frequency, all analyses were performed by use of conditional methods that implicitly adjust for matching. The serosurvey number was used as the matching variable to condition each case-control set. A child was sampled only once within each 3-month interval, and there were no repeated measures for a child for the related serosurvey. Because this is a conditional analysis in which estimation is performed by conditioning on the serosurvey, adjustment for repeated measures was not performed. To assess the distribution of the baseline variables (e.g., age and sex) among the case and control subjects, simple 2-group comparisons were performed by use of conditional logistic regression with a single independent variable and with multiple case and control subjects per stratum [27]. Serum antibody titers were log transformed, to obtain normal distributions. To examine whether a dose-response relationship existed between antibody titers and protection against disease and to evaluate whether a threshold antibody-titer range existed above which disease did not occur, serum antibody titers were categorized into quintiles on the basis of their distribution in the control group. These quintiles were used to classify the distribution of the serum antibody titers for the case subjects. To compute the odds ratio (OR) for each category of serum antibody titer, the lowest quintile of the control group was chosen as the reference category.

Because age was a strong predictor of case-control status, it was necessary to adjust for the effect of age in the evaluation of the relationship between antibody titers and protection. To account for matching and simultaneously adjust for the effect of age, we used multivariable conditional logistic regression.

Table 2. Characteristics of case subjects who experienced an enterotoxigenic *Escherichia coli* (ETEC) diarrheal episode during which colonization factor antigen (CFA)/I or heat-labile enterotoxin (LT) was expressed, compared with those of their matched control subjects.

	CFA/I		LT[a]	
Characteristic	Case subjects (n = 21)	Control subjects (n = 154)	Case subjects (n = 51)	Control subjects (n = 280)
Age[b]				
9–11 months	7 (33)	24 (3)	13 (25)	19 (7)
12–17 months	10 (48)	28 (18)	15 (28)	48 (17)
18–23 months	3 (14)	33 (21)	9 (18)	64 (23)
⩾24 months	1 (5)	89 (58)	14 (27)	149 (53)
Maternal age, mean ± SD, years	24 ± 5	26 ± 8	25 ± 5	26 ± 7
Breast-fed[c]	17 (81)	43 (28)[d]	25 (49)	81 (29)[e]
Males	11 (52)	71 (46)	26 (51)	130 (46)
Village 830[f]	14 (67)	75 (49)	30 (59)	134 (48)
Serosurvey				
1	0 (0)	0 (0)	10 (20)	55 (20)
2	2 (10)	15 (10)	8 (16)	40 (14)
3	16 (76)	114 (74)	13 (25)	75 (27)
4	3 (14)	25 (16)	13 (25)	75 (27)
5	0 (0)	0 (0)	7 (14)	35 (13)
Maternal education[g]	2 (10)	13 (9)	5 (10)	14 (5)
Flush toilet	1 (5)	9 (6)	6 (12)	14 (5)
Electricity	18 (86)	132 (86)	47 (92)	244 (87)
Ownership of livestock	16 (7)	128 (83)	42 (82)	225 (80)
Crowding[h]	7 (33)	50 (32)	18 (35)	97 (35)
Luxury[i]	18 (86)	126 (82)	46 (90)	232 (83)

NOTE. Data are no. (%) of subjects, unless otherwise noted.

[a] Refers to LT-ETEC diarrheal episodes during which CF was not expressed.
[b] Age of case and control subjects on the date that blood was obtained.
[c] Child was breast-fed at the time of the serosurvey.
[d] $P < .0001$.
[e] $P < .01$.
[f] Resident of village 830, 1 of the 2 villages under study.
[g] Mother received some formal education.
[h] Crowding in the house, defined as >5 persons/sleeping room.
[i] Ownership of ⩾1 luxury item (e.g., television, water heater, electric oven, motorcycle, refrigerator, or washing machine).

To determine the antibody-titer range above which protection is observed, a stepwise conditional logistic regression model was used; in the model, the antibody-titer quintiles were coded as dummy variables by use of a modified coding strategy, to reflect the ordinal nature of the antibody titers [28]. Age and the variables representing the titer quintiles were stepped into the model by use of the forward-selection strategy. The antibody-titer variable that first enters the model in the selection process and contributes to the best improvement of the fit of the model in predicting case-control status can be interpreted as the optimal titer that may be associated with protection against the disease outcome [28].

RESULTS

Between July 1996 and October 1997, a total of 5 serosurveys were conducted. Of 1045 blood specimens scheduled to be drawn, 1025 (98%) were successfully obtained. Active surveillance detected 344 children who experienced ETEC diarrheal episodes, of which 56 were excluded as case subjects because they were coinfected with another pathogen and 6 were excluded as case subjects because the episodes occurred during the 3-day window after the blood was obtained. Of the remaining 282 children, the expression of LT, CFA/I, CFA/II, and CFA/IV was detected in specimens from 65, 32, 29, and 31, respectively. Because some specimens had to be tested for multiple antigens and because each specimen was tested twice on different days, there were insufficient quantities of serum available from some case subjects for replicate testing. Sufficient quantities of serum were available from 51, 21, 22, and 23 of the case subjects who experienced LT-, CFA/I-, CFA/II-, and CFA/IV-ETEC diarrheal episodes, respectively. Corresponding to these case subjects, there were 325, 157, 141, and 135 specimens from the matched control subjects; sufficient quantities of serum were available for replicate testing from 280, 154, 112,

Table 3. Serum antibody titers for case subjects who experienced an enterotoxigenic *Escherichia coli* (ETEC) diarrheal episode during which colonization factor antigen (CFA)/I, CFA/II, CFA/IV, or heat-labile enterotoxin (LT) was expressed, compared with those of their matched control subjects.

Target antigen, age group	Case subjects	Control subjects
CFA/I		
All[a]	2.16 ± 0.70 (21)	2.49 ± 0.53 (154)
Age[b]		
9–11 months	1.71 ± 0.36 (7)	1.97 ± 0.59 (4)
12–17 months	2.17 ± 0.68 (10)	2.55 ± 0.64 (28)
18–23 months	2.90 ± 0.74 (3)	2.50 ± 0.67 (33)
⩾24 months	2.95 ± 0 (1)	2.49 ± 0.41 (89)
CFA/II (CS3)		
All	2.29 ± 0.66 (22)	2.24 ± 0.67 (112)
Age		
9–11 months	1.57 ± 0.01 (2)	2.40 ± 1.05 (4)
12–17 months	2.10 ± 0.68 (6)	2.04 ± 0.77 (17)
18–23 months	2.57 ± 0.72 (5)	2.10 ± 0.61 (25)
⩾24 months	2.43 ± 0.59 (9)	2.33 ± 0.63 (66)
CFA/IV (CS6)		
All	2.10 ± 0.72 (23)	2.39 ± 0.69 (92)
Age		
9–11 months	1.40 ± 0.27 (3)	1.76 ± 0.43 (7)
12–17 months	1.87 ± 0.70 (4)	2.31 ± 0.87 (12)
18–23 months	1.96 ± 0.41 (3)	2.39 ± 0.76 (19)
⩾24 months	2.37 ± 0.75 (13)	2.50 ± 0.62 (54)
LT_B[c]		
All	3.64 ± 0.42 (51)	3.60 ± 0.37 (280)
Age		
9–11 months	3.66 ± 0.64 (13)	3.69 ± 0.46 (19)
12–17 months	3.79 ± 0.29 (15)	3.69 ± 0.36 (48)
18–23 months	3.57 ± 0.37 (9)	3.71 ± 0.39 (64)
⩾24 months	3.52 ± 0.31 (14)	3.51 ± 0.33 (149)

NOTE. Data are mean ± SD log antibody titers (no. of subjects). CS, coli surface antigen.

[a] $P < .05$.

[b] $P < .05$, for case subjects vs. control subjects <18 months of age.

[c] Refers to LT-ETEC diarrheal episodes in which the isolated ETEC strain(s) did not express a CF.

and 92 of the control subjects, respectively. The baseline demographic characteristics of the case and control subjects from whom insufficient quantities of serum were available did not differ from those of the case and control subjects whose serum specimens were tested.

The clinical features of the case subjects whose serum specimens were tested are presented in table 1. CFA/I-ETEC diarrheal episodes occurred at younger ages and included more severe symptoms than did LT-, CFA/II-, and CFA/IV-ETEC diarrheal episodes, as was evidenced by the longer duration of disease, the greater number of loose stools, and the greater likelihood of treatment with oral rehydration solution in the case subjects who experienced CFA/I-ETEC diarrheal episodes (table 1). Of the 22 case subjects who experienced CFA/II-ETEC diarrheal episodes, 10 (45%) had isolates that expressed both CS1 and CS3, and 6 (27%) had isolates that expressed both CS2 and CS3. Of the 23 case subjects who experienced CFA/IV-ETEC diarrheal episodes, 22 (96%) had isolates that expressed CS6 alone, and 1 (4.3%) had an isolate that expressed both CS5 and CS6.

The case subjects did not differ from the control subjects with respect to socioeconomic status or maternal characteristics. However, the case subjects who experienced CFA/I- and LT-ETEC diarrheal episodes were significantly younger than their matched control subjects (table 2). Because of this age distribution, these case subjects were more likely to be breastfed than their matched control subjects (table 2). For the CFA/II- and CFA/IV-ETEC groups, the age distribution and socioeconomic status of the case subjects did not differ from those of the matched control subjects (data not shown). For CFA/I antibodies, the mean log-transformed titers for the case subjects were significantly lower than those for the matched control subjects (reciprocal geometric mean titers, 145 vs. 309) ($P < .05$) (table 3). This pattern was not, however, consistent across the different age groups for CFA/I antibodies. For children ⩾18 months of age, the mean antibody titer for the case subjects who experienced CFA/I-ETEC diarrheal episodes was higher than that for the matched control subjects, although the difference was not statistically significant. In contrast, for children <18 months of age, the case subjects who experienced CFA/I-ETEC diarrheal episodes had significantly lower antibody titers than did the matched control subjects (table 3). For the LT-, CFA/II-, and CFA/IV-ETEC case-control groups, the overall and age-specific log-transformed mean antibody titers for the case subjects did not differ from those for the matched control subjects (table 3).

The ORs for the different levels of CS3 and LT_B antibodies in serum suggest that serum antibody titers are not related to the odds of disease (table 4). In contrast, for CFA/I antibodies, the odds of disease were significantly lower for higher antibody levels, compared with the lowest level; also, on the basis of the magnitude of the ORs, there was a suggestive association for CS6 antibodies. In crude analyses, for CFA/I antibodies there appeared to be a threshold effect as opposed to a dose-response relationship, with reduced ORs observed for all reciprocal antibody titers >100. However, on the basis of the adjusted ORs, there was a graded increase in protective associations, with increasing reciprocal titers up to 813 and then a paradoxical absence of such an association at higher reciprocal titers (table 4). Although the relationship between CFA/IV-ETEC disease and titers of CS6 antibodies was not statistically significant, the magnitude of the ORs suggests that such an association might exist (table 4). The apparent lack of association between titers of CFA/I antibodies and disease after adjustment for age ($P = .35$) can be attributed to a statistically significant interaction between age and titers of CFA/I antibodies ($P < .05$). A scatter

Table 4. Dose-response relationship between serum antibody titers and the odds of disease, in case subjects who experienced an enterotoxigenic *Escherichia coli* (ETEC) diarrheal episode during which colonization factor antigen (CFA)/I, CFA/II, CFA/IV, or heat-labile enterotoxin (LT) was expressed.

Target antigen, antibody titers	No. (%) of subjects		OR (95% CI)	
	Case subjects	Control subjects	Crude	Adjusted[a]
CFA/I				
<99	11 (52)	30 (19)	1	1
99–244	3 (14)	31 (20)	0.3 (0.1–1.0)	0.8 (0.2–3.7)
245–426	1 (5)	30 (19)	0.1 (0.01–0.7)[b]	0.3 (0.03–2.9)
427–812	1 (5)	31 (20)	0.1 (0.01–0.7)[b]	0.2 (0.02–1.8)
⩾813	5 (24)	32 (21)	0.5 (0.1–1.6)	1.6 (0.3–8.2)
Total	21	154	…	…
CFA/II (CS3)				
<44	5 (23)	22 (20)	1	1
44–104	4 (18)	22 (20)	0.6 (0.1–2.7)	1.0 (0.2–5.3)
105–185	3 (14)	23 (21)	0.5 (0.1–2.6)	0.9 (0.2–4.8)
186–616	4 (18)	22 (20)	0.7 (0.2–3.3)	1.4 (0.3–6.9)
⩾617	6 (27)	23 (21)	1.4 (0.3–5.8)	2.4 (0.5–11.4)
Total	22	112	…	…
CFA/IV (CS6)				
<60	9 (39)	18 (20)	1	1
60–131	4 (17)	18 (20)	0.4 (0.1–1.6)	0.5 (0.1–1.8)
132–315	4 (17)	19 (21)	0.4 (0.1–1.6)	0.5 (0.1–1.9)
316–976	3 (13)	17 (18)	0.4 (0.1–1.6)	0.5 (0.1–2.1)
⩾977	3 (13)	20 (22)	0.3 (0.1–1.4)	0.4 (0.1–1.9)
Total	23	92	…	…
LT_B[c]				
<1950	8 (16)	56 (20)	1	1
1950–2883	9 (18)	51 (18)	1.2 (0.4–3.5)	1.2 (0.4–3.5)
2884–4897	12 (24)	61 (22)	1.4 (0.5–3.7)	1.5 (0.5–4.0)
4898–7942	8 (16)	55 (20)	1.0 (0.4–3.0)	0.8 (0.3–2.2)
⩾7943	14 (27)	57 (20)	1.8 (0.7–4.6)	1.2 (0.4–3.2)
Total	51	280	…	…

NOTE. CI, confidence interval; OR, odds ratio.

[a] Adjusted for age.

[b] $P < .05$.

[c] Refers to LT-ETEC diarrheal episodes during which CFA was not expressed.

plot of age and titers of CFA/I antibodies with a nonparametric smooth curve fit separately to case subjects, control subjects, and case and control subjects combined illustrates this statistical interaction (figure 1). Because of this interaction, subgroup analysis by age was necessary to evaluate the association between antibody titers and case-control status. For CFA/I antibodies, the log mean antibody titers were lower for the case subjects <18 months of age, compared with those for the matched control subjects ($P < .05$), but were higher for the case subjects ⩾18 months of age, compared with the matched control subjects ($P = .09$) (table 3). On the basis of this observation and the observation that the 2 curves for titers of CFA/I antibodies for the case and control subjects shown in figure 1 intersect at 18 months of age, a cutoff age of 18 months was chosen to evaluate the association between case-control status and serum antibody titers within the 2 age groups. For children <18 months of age, the association between serum antibody titers and case-control status did not appear to have a dose-response relationship (table 5), and all serum antibody titers greater than the referent level revealed ORs <1. The stepwise regression model selected the serum antibody-titer range of 76–186 to be the most significant variable associated with case-control status, followed by age. After adjustment for age, the children with serum antibody titers ⩾76 had 77% (95% confidence interval, 13%–94%; $P < .05$) lower odds of being a case subject, compared with the children with serum antibody titers <76. Overall, there were no significant relationships between titers of CS3, CS6, or LT_B antibodies and age.

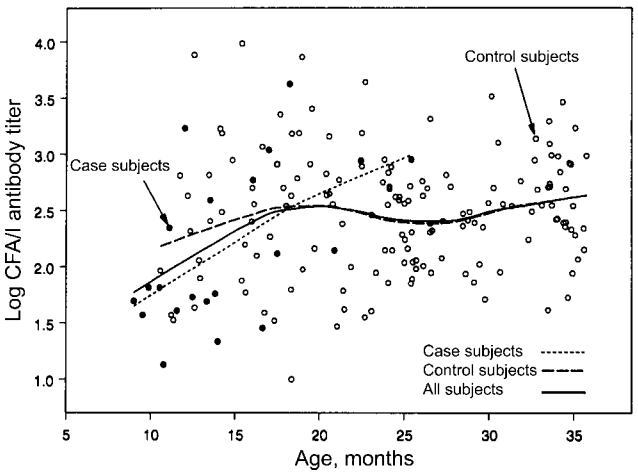

Figure 1. Scatter plot of age in months vs. titers of colonization factor antigen (CFA)/I antibodies with a nonparametric smooth curve fit separately for case subjects *(dotted line),* control subjects *(dashed line),* and case and control subjects combined *(solid line).*

DISCUSSION

The present study was conducted to assess the extent to which serum levels of specific CF or LT antibodies may serve as predictors or markers of protective immunity. The resultant data indicate that, among Egyptian children 9–17 months of age, serum levels of IgG antibody against CFA/I are good immune markers of protection against disease caused by ETEC strains expressing this CF; among these children, there appeared to be protection against CFA/I-ETEC diarrhea for those with reciprocal antibody titers ⩾76.

Previous studies have shown that serum antibody levels correlate with protection against diarrheal illness caused by cholera and rotavirus [29–33]. Serum vibriocidal antibodies against *Vibrio cholerae* O1 have also been shown to correlate with the intestinal immunity that is acquired through exposure to natural infection [34]. However, little is known about the relationship between natural infection, intestinal immunity, serum antibody levels, and the risk of diarrhea due to ETEC infection. A study in children presenting to clinics for treatment of ETEC diarrhea in rural Bangladesh reported that titers of CFA/I and CFA/II antibodies increased 8-fold from the acute to the convalescent periods of their episodes [24]. This observation suggests that there may be intestinal secretory IgA antibody responses and corresponding serum IgG antibody responses to an acute ETEC infection. However, case-control comparisons in the same study found no association between the risk of infection with ETEC and levels of homologous serum antibodies against LT, CFA/I, or CFA/II. A seroepidemiologic study conducted in a cohort of rural Argentinean children for whom multiple cross-sectional serosurveys were conducted reported increasing levels of antibodies against LT and CFA/II over the course of follow-up during the first 2 years of life. Here, too, no apparent relationship was observed between the serum levels of LT or CF antibodies in these children and a reduced risk of acquiring homologous ETEC diarrhea.

In the present study, we did not find an association between serum levels of IgG antibody against LT_B and the risk of LT-ETEC diarrhea, a finding similar to those reported in the Argentina and Bangladesh studies [24, 35]. Others, too, have failed to detect an inverse association between the risk of cholera and serum levels of antibody against the B subunit of cholera toxin (CT_B), which is highly similar to LT_B [29, 30]. This contrasts with clinical-trial findings demonstrating the protective efficacy of oral vaccination with CT_B against LT-ETEC diarrhea [14, 36].

Most consequential are our results suggesting a substantial reduction in risk of CFA/I-ETEC diarrhea for children with high levels of IgG antibodies against this CF, a finding different from that reported in the Bangladesh study [24]. The differences in the designs of these 2 studies may account for the discrepant findings. In the Bangladesh study, acute serum specimens were collected shortly after the onset of symptoms of ETEC diarrhea; as a result, the measured antibodies may be

Table 5. Dose-response relationship between serum antibody titers and the odds of disease, in case subjects <18 months of age who experienced an enterotoxigenic *Escherichia coli* diarrheal episode during which colonization factor antigen/I was expressed.

	No. (%) of subjects		OR (95% CI)	
Antibody titers	Case subjects	Control subjects	Crude	Adjusted[a]
<76	11 (64)	7 (21)	1	1
76–185	1 (6)	6 (19)	0.1 (0.01–1.1)	0.15 (0.02–1.5)
186–446	2 (12)	6 (19)	0.25 (0.04–1.5)	0.3 (0.04–1.6)
447–999	1 (6)	7 (21)	0.1 (0.01–1.0)	0.2 (0.02–1.7)
⩾1000	2 (12)	6 (19)	0.6 (0.04–8.6)	0.6 (0.03–8.9)

NOTE. CI, confidence interval; OR, odds ratio.

[a] Adjusted for age.

more reflective of an anamnestic response to ETEC infection and, hence, may not accurately reflect true preinfection antibody levels in the case subjects.

In the present study, reduced odds of CFA/I-ETEC diarrhea with higher levels of specific antibodies was noted for the subgroup of case subjects 9–17 months of age but not for the older children. This observation may be attributed to the age distribution of case and control subjects in the study population and the decreasing incidence of CFA/I-ETEC diarrhea with age. Protection was associated with reciprocal titers in the 76–186 range. Because there were only 17 case subjects <18 months of age who experienced CFA/I-ETEC diarrheal episodes, the findings may be sensitive to variation in the cutoff levels; hence, it was not possible to provide a single threshold associated with protection. Larger studies are needed to confirm these findings and to provide a more definitive threshold. The observation that the case subjects >18 months of age had a higher geometric mean titer than did the control subjects of the same age may be a result of chance, because it was based on small numbers. There were only 4 case subjects who experienced CFA/I-ETEC diarrhea, of which only 1 case subject was ⩾24 months of age. In contrast, nearly 60% of the control subjects were ⩾24 months of age (table 2). Our inability to perform further statistical analysis within this subgroup of older children was limited by this parsimonious distribution.

We failed to find an association between the risk of CFA/II- and CFA/IV-ETEC diarrhea and serum levels of antibodies against CS3 and CS6, respectively. This, however, does not necessarily imply that mucosal immunity against CFA/II- and CFA/IV-ETEC diarrhea is not relevant to protection. It is possible that serum levels of IgG antibodies against the common CFA/II (i.e., CS3) and CFA/IV (i.e., CS6) antigens are not good correlates of protection against disease in the present population. Although our data suggest that there was no association between CS3 antibody levels and the odds of CFA/II-ETEC diarrhea, CS6 antibody levels appeared to be more predictive of some degree of immunity against CFA/IV-ETEC diarrhea. In light of the magnitude of the adjusted ORs of ~0.35 for the different levels of antibodies (table 4), it could be stated that an association between CS6 antibody levels and protection might indeed exist but that our study was underpowered to detect it. Antibodies against CS1 and CS2 may play an important role in protection against CFA/II-ETEC diarrhea, especially in settings like ours where the majority of isolates from children who experienced a CFA/II-ETEC diarrheal episode expressed one of these antigens in conjunction with CS3. Limitations in the amount of serum available for testing precluded an evaluation of this hypothesis in the present study. Another possible explanation for the negative associations observed is that the ELISAs used may not have captured responses to the as-yet undefined protective epitopes of these complex antigens.

In conclusion, the present study has found that serum levels of CFA/I antibodies in young children show an inverse association with the subsequent risk of CFA/I-ETEC diarrhea. We speculate that candidate vaccines capable of inducing reciprocal serum titers of IgG antibody against CFA/I within or above the 76–186 range in pediatric populations may prove more likely to confer protection in efficacy trials.

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